Correlation of NNK levels in tobacco and moist snuff with the levels of pseudooxynicotine and nicotine-1’-N-oxide

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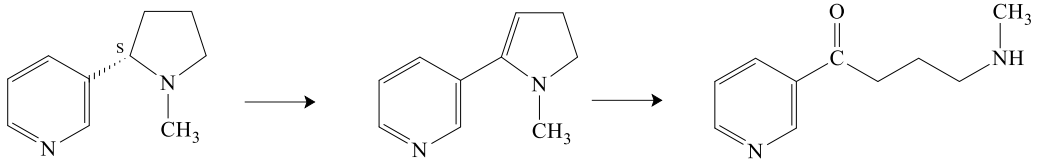
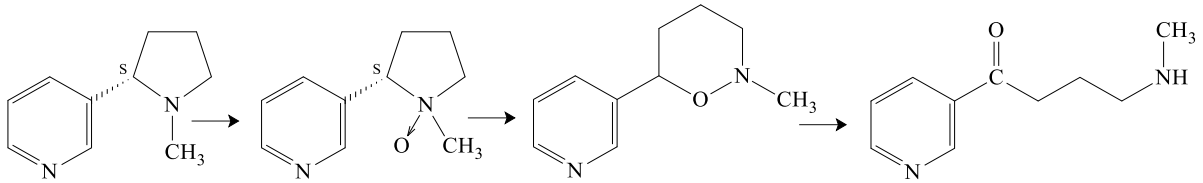
Background

- Nicotine can be metabolized by various pathways by different microorganisms. One such pathway caused by bacteria of the genus *Pseudomonas* and the fungus *Cunninghamella echinulata* degrade nicotine with the formation of N-methylmyosmine and further formation of pseudooxynicotine (PON):



Nicotine N-methylmyosmine PON

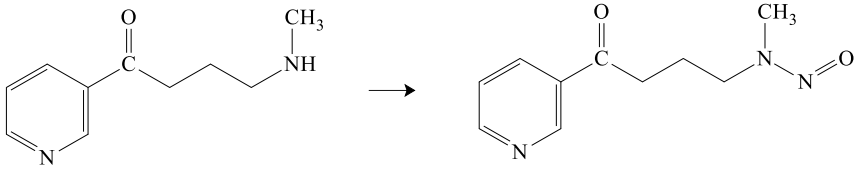
- Oxidation of nicotine caused by flavin-containing monooxygenases generates nicotine-1'-N-oxide, the compound being also obtained from non-enzymatic nicotine oxidation. Nicotine-1'-N-oxide (Nic-Ox) can be isomerized by heating to form 2-methyl-6-(pyridine-3-yl)-1,2-oxazinane that can also lead to the formation of pseudooxynicotine (PON):



Nicotine Nicotine-1'-N-oxide PON

Background

- By nitrosation, pseudooxynicotine can generate 3-(N-methyl-N-nitrosamino)propyl 3-pyridyl ketone (NNK) by the following reaction:



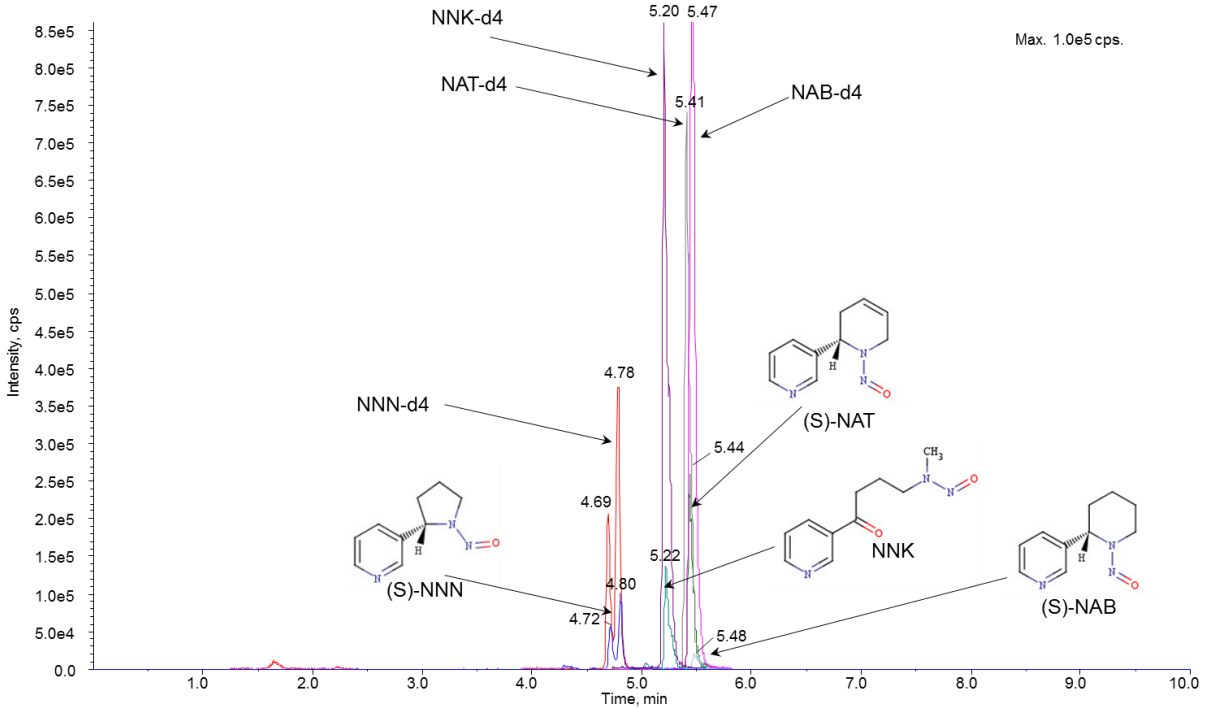
PON NNK

- NNK is a potentially carcinogenic tobacco specific nitrosamine (TSNA) and significant effort is being made to reduce its level in tobacco products.
- The elevated levels of nicotine-1'-N-oxide and of pseudooxynicotine in tobacco may be a source for the NNK in tobacco [Y. Wu, H. Ji, A. Fisher, L. Bush, Relationship of alkaloids, pseudooxynicotine, tobacco-specific nitrosamines, nitrate/nitrite, from two-year field analysis, 73rd Tobacco Research Conference, vol 73, Leesburg, VA, Sept. 15-18, 2019, Presentation 52.].
- In order to test this hypothesis, in this study the levels of NNK, of nicotine-1'-N-oxide (Nic-Ox), and of pseudooxynicotine (PON) were measured in a number of tobacco samples and in several commercial moist snuff samples.
- The correlation between PON levels and NNK levels as well as the correlation between Nic-Ox levels and NNK levels were evaluated.

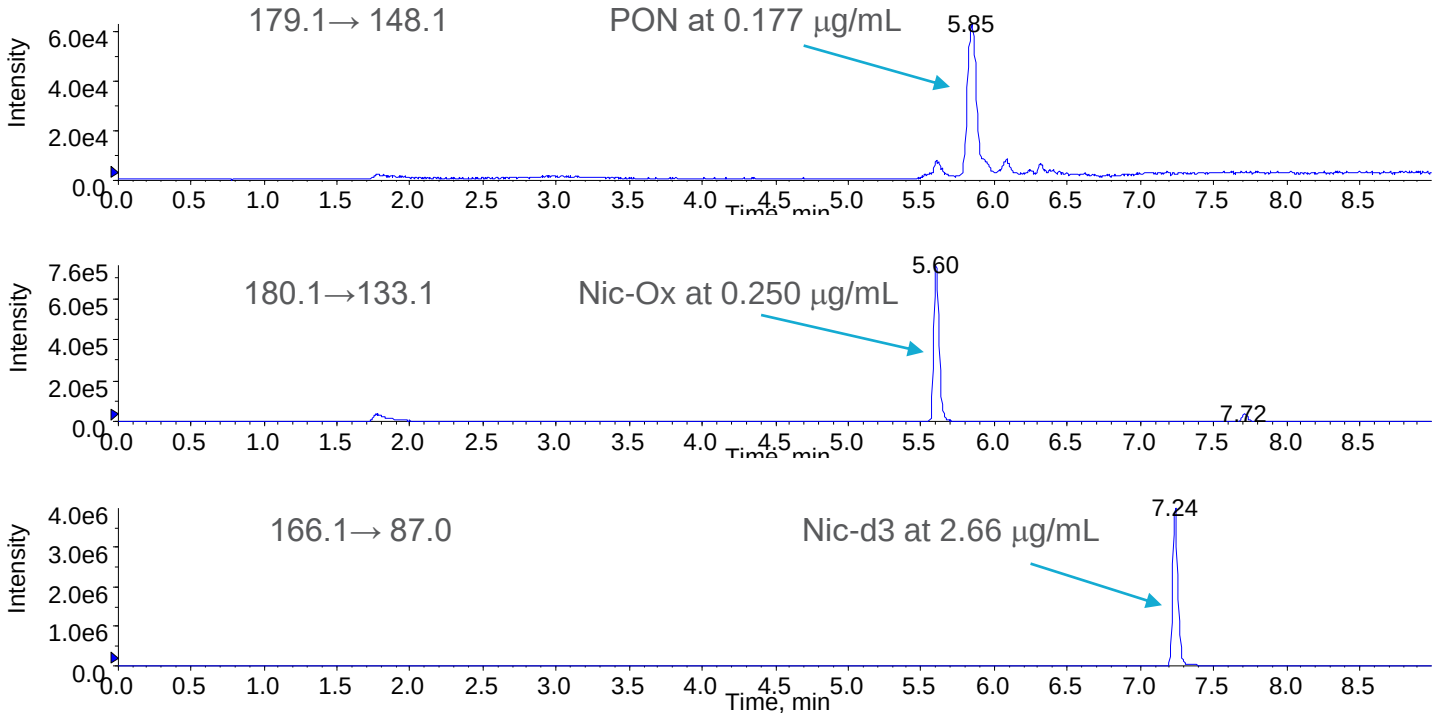
Summary of analytical procedure

- Three analytical procedures were used in the study, one for nicotine level, one for PON and Nic-Ox, and one for TSNAs measurements.
- The nicotine was measured using a HPLC with UV detection procedure.
- The TSNAs were measured using a LC-MS/MS procedure reported in the literature [S. C. Moldoveanu, M. Adams, F. K. St.Charles, Variations of TSNA levels in tobaccos upon heating at moderate temperatures, *Beitr. Tabakforsch. Intern*., 29/2 (2020) 84-96].
- PON and Nic-Ox were analyzed using separation on an Acquity UPLCTM BEH Phenyl 1.7 μm column, 30 x 150 mm dimensions for Waters. The mobile phase was made from solvent A: 0.02 M NH_4COOH in water brought to pH 9.5 with NH_4OH, and solvent B: acetonitrile in gradient conditions.
- The detection of PON and Nic-Ox was done using ESI-MS/MS working in positive MRM mode.
- The quantitation of PON and Nic-Ox was done using calibration curves peak area vs. standard concentration or normalized peak area (by the peak of the standard) vs. peak area of nicotine-d_3 (at 2.66 μg/mL). Seven standards were used for calibration in the range 0.022 – 1.419 μg/mL for PON and in the range 0.031 – 2.000 μg/mL for Nic-Ox.
- LOQ for both compounds can be taken as equal with the value of the lowest standard, although based on signal to noise (S/N) values, a much lower LOQ would be obtained.

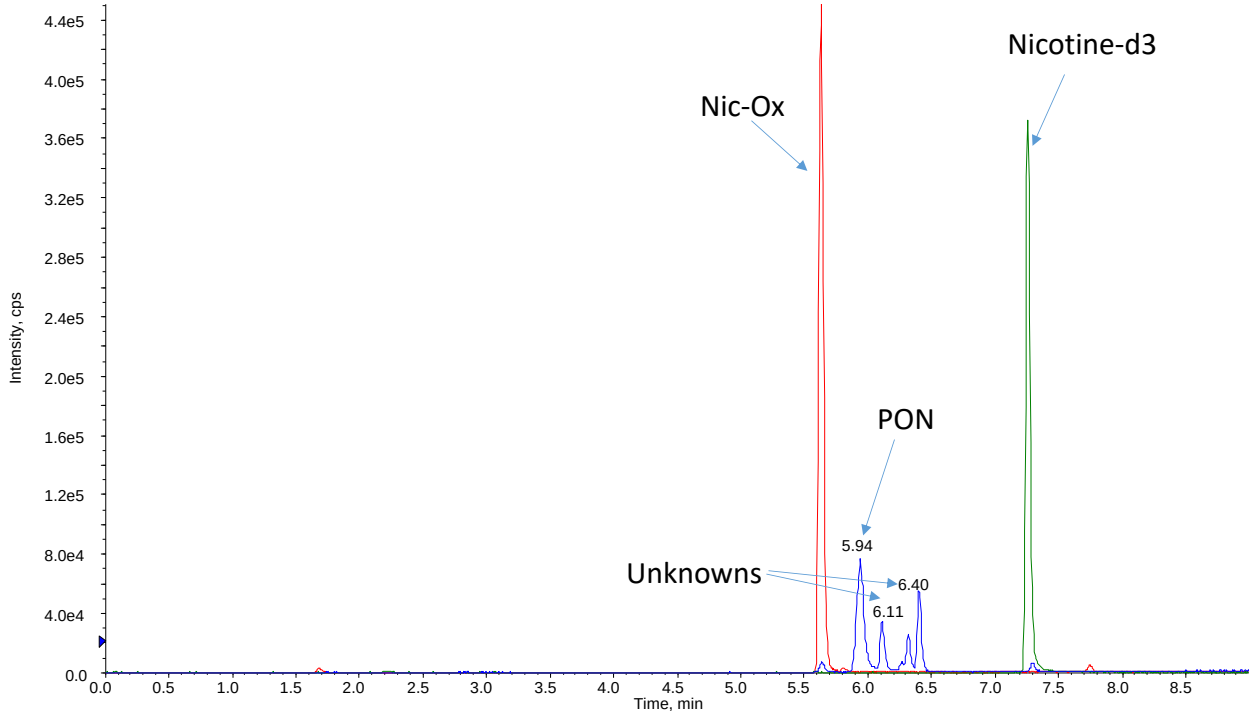
Extracted MRM traces for TSNAs from a flue-cured tobacco sample (syn/anti forms seen for some peaks)



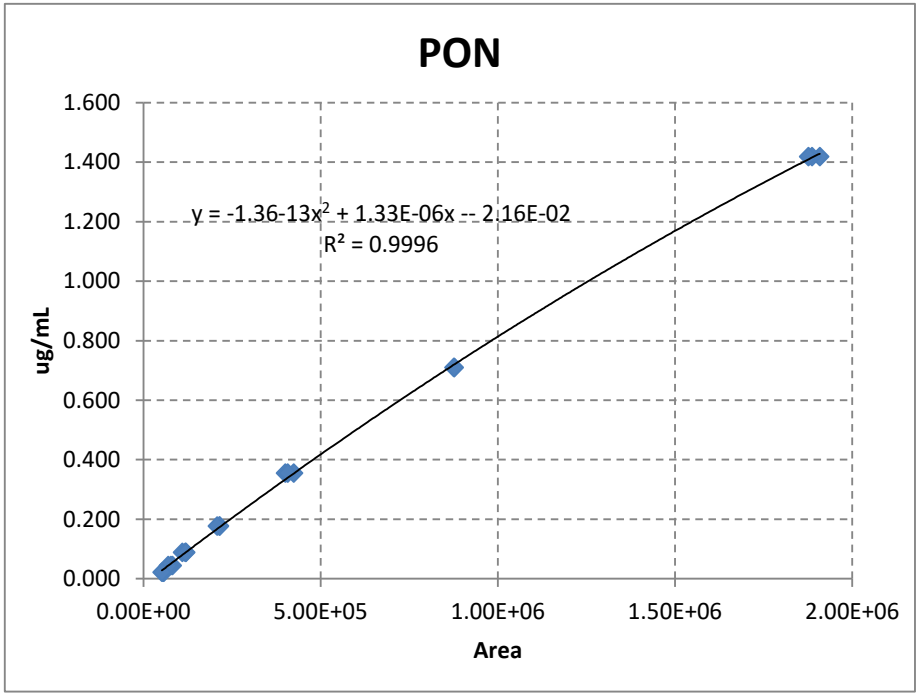
Extracted MRM traces for a standard of PON and Nic-Ox

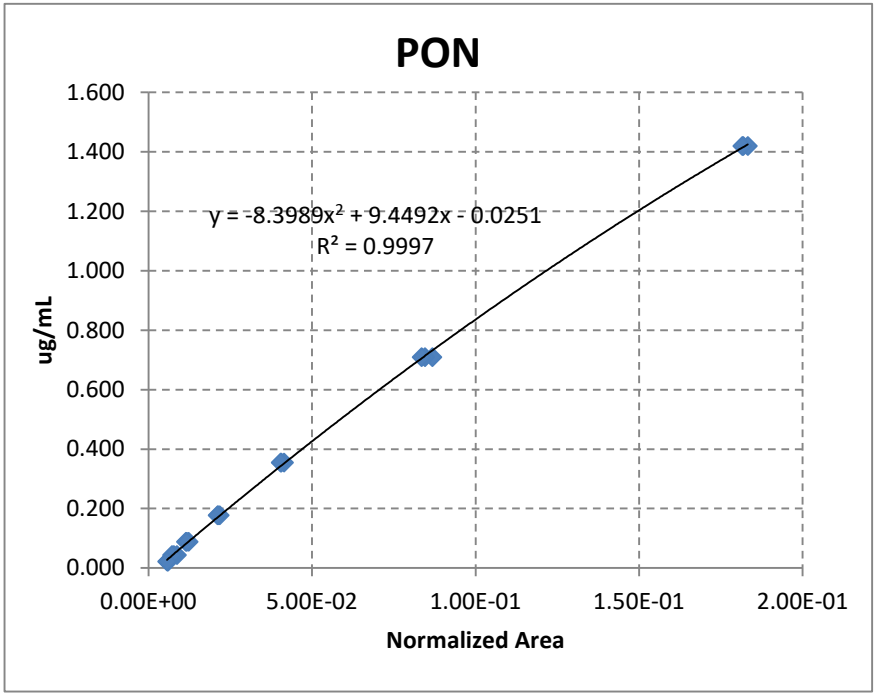


Extracted MRM traces for PON and Nic-Ox from a burley tobacco sample

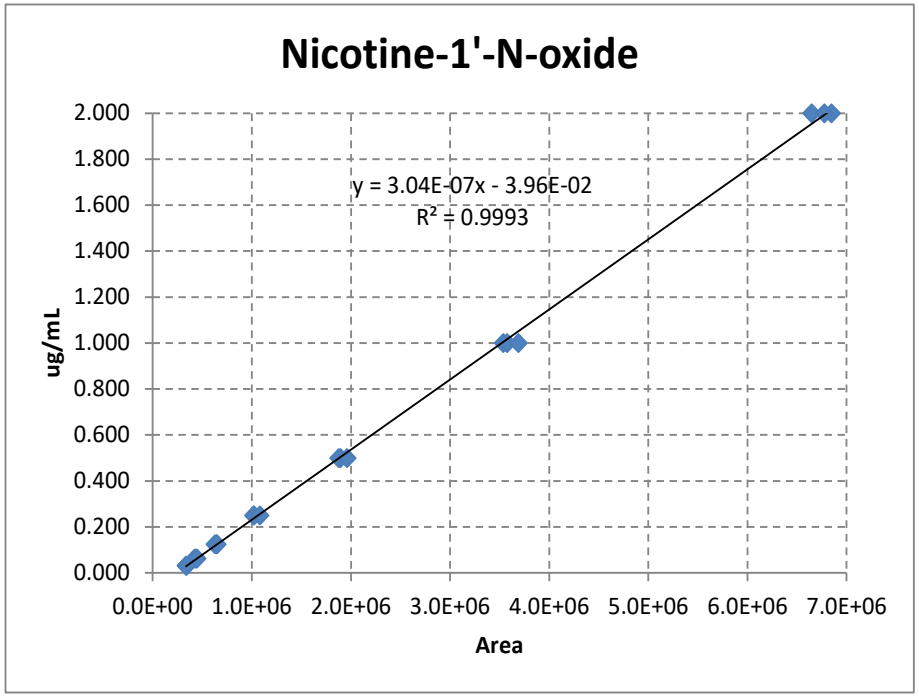


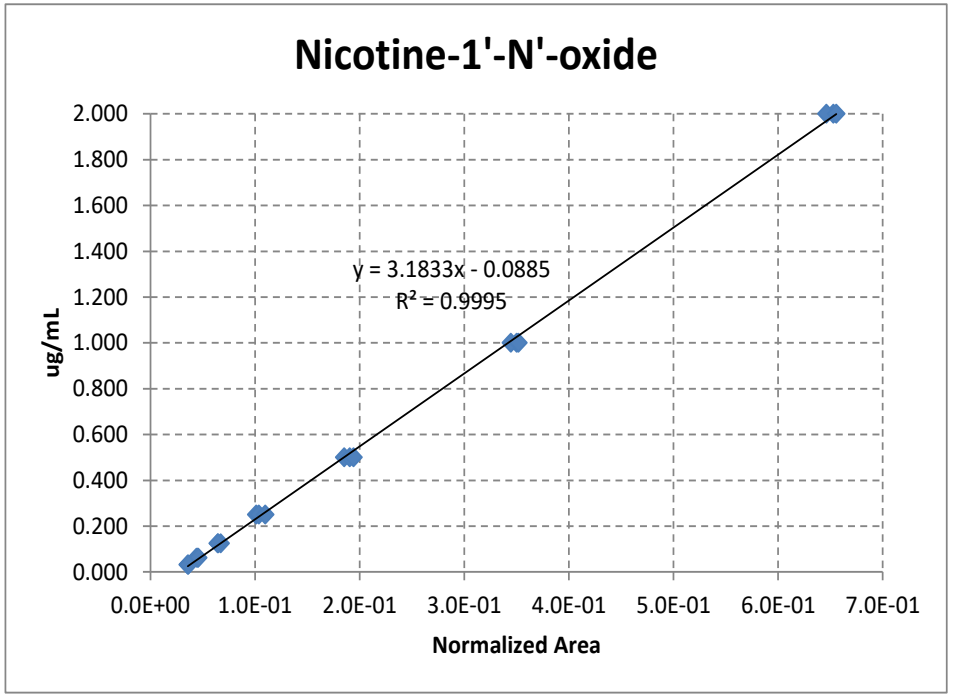
Calibration curves for PON





Calibration curves for Nic-Ox





Samples evaluated in the study (1)

No.	Sample	Description
1	FC L (1)	Eastern NC belt, lower stalk (lug) flue-cured (2016)
2	FC U (1)	Eastern NC belt, upper stalk (leaf & some tips) flue-cured (2016)
3	FC L (2)	South Carolina belt, lower stalk (lug) flue-cured (2016)
4	FC U (2)	South Carolina belt, upper stalk (leaf & some tips) flue-cured (2016)
5	FC off L	Brazil, lower stalk (lugs & primings) flue-cured (2016)
6	FC off U	Brazil, upper stalk (leaf & tips) flue-cured (2016)
7	Bu L (1)	Kentucky & Tennessee, lower stalk (flyings & cutters) burley (2016)
8	Bu U (1)	Kentucky & Tennessee, upper stalk (leaf) burley (2016)
9	Bu L (2)	North Carolina & Virginia, lower stalk (flyings & cutters) burley (2016)
10	Bu U (2)	North Carolina & Virginia, upper stalk (leaf) burley (2016)
11	Bu off L	Malawi, lower stalk (flyings & cutters) burley (2016)
12	Bu off U	Malawi, upper stalk (leaf) burley (2016)
13	O SA U	Turkey, good quality middle to upper stalk, Samsun oriental (2015)
14	O Iz U	Turkey, good quality middle to upper stalk, Izmir oriental (2015)
15	FC L-Prod	Low stalk flue-cured blend used in cigarette production (2018)

Samples evaluated in the study (2)

No.	Sample	Description
16	FC U-Prod	Leaf flue-cured blend used in cigarette production (2018)
17	Bu L-Prod	Low stalk burly blend used in cigarette production (2018)
18	Bu U-Prod	Leaf burley blend used in cigarette production (2018)
19	O1-Prod	Oriental blend used in cigarette production (2018)
20	O2-Prod	Oriental blend used in cigarette production (2018)
21	FC1-LN	Low nicotine flue-cured blend (2018)
22	FC2-LN	Low nicotine flue-cured blend (2018)
23	Bu-LN	Low nicotine burley (2018)
24	Moist 1	Natural Fine Cut (2019)
25	Moist 2	Long Cut Wintergreen (2019)
26	Moist 3	Natural Fine Cut (2019)
27	Moist 4	Fine Cut Wintergreen (2019)
28	Moist 5	Long Cut Wintergreen (2019)
29	Moist 6	Wintergreen (2019)
30	Moist 7	Natural Fine Cut (2019)
31	Moist 8	Long Cut Mint (2019)

Results for PON, Nic-Ox and NNK (1)

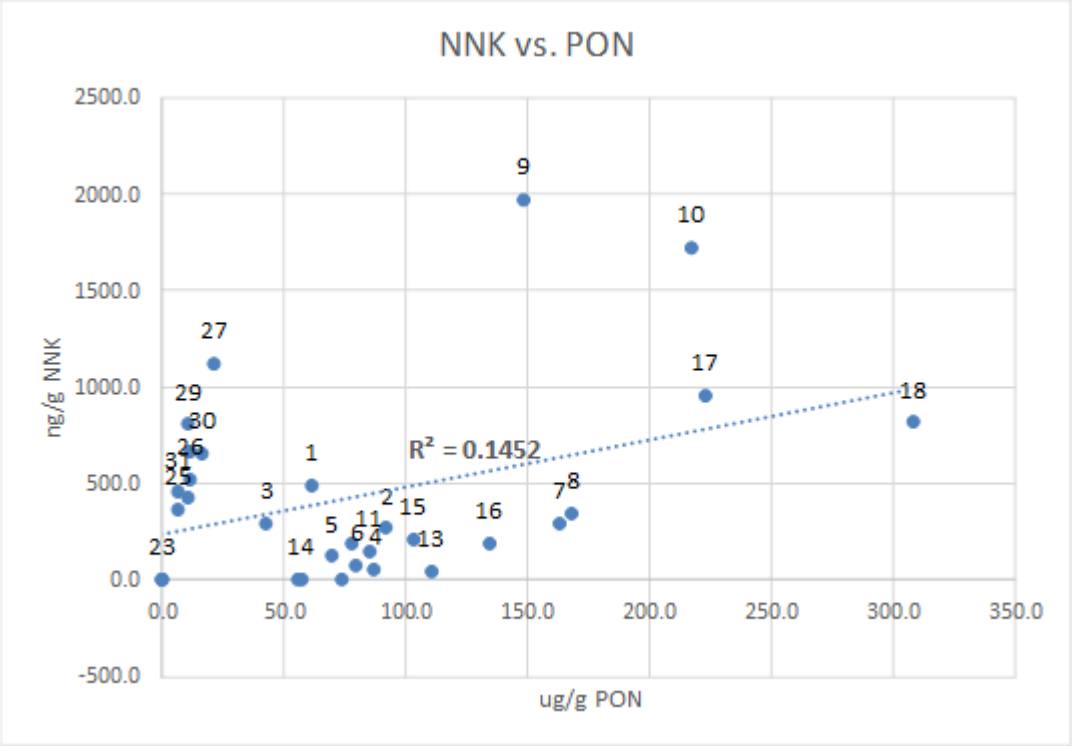
No.	Sample	PON mg/g	RSD%	Nic-Ox mg/g	RSD%	NNK ng/g	RSD%
1	FC L (1)	61.4	0.3	113.0	1.0	492.6	1.96
2	FC U (1)	91.9	0.7	149.3	0.5	268.8	0.53
3	FC L (2)	43.0	0.9	56.1	0.9	295.4	0.60
4	FC U (2)	87.2	0.0	71.1	0.7	56.1	0.20
5	FC off L	69.3	0.9	164.0	0.7	122.4	0.71
6	FC off U	79.7	0.4	203.0	0.2	80.3	0.04
7	Bu L (1)	163.0	0.4	244.1	0.4	298.2	0.25
8	Bu U (1)	168.2	0.5	275.2	0.1	344.0	0.09
9	Bu L (2)	148.3	0.6	230.9	0.9	1975.8	1.98
10	Bu U (2)	217.0	0.1	266.0	0.6	1724.9	2.12
11	Bu off L	85.2	0.1	138.7	0.1	152.7	0.88
12	Bu off U	77.7	0.1	123.3	0.4	194.7	0.40
13	O SA U	110.6	0.7	245.5	0.8	48.1	0.26
14	O Iz U	57.3	0.9	157.8	1.3	0.6	7.00
15	FC L-Prod	103.0	0.5	224.1	1.2	214.1	1.46

Results for PON, Nic-Ox and NNK (2)

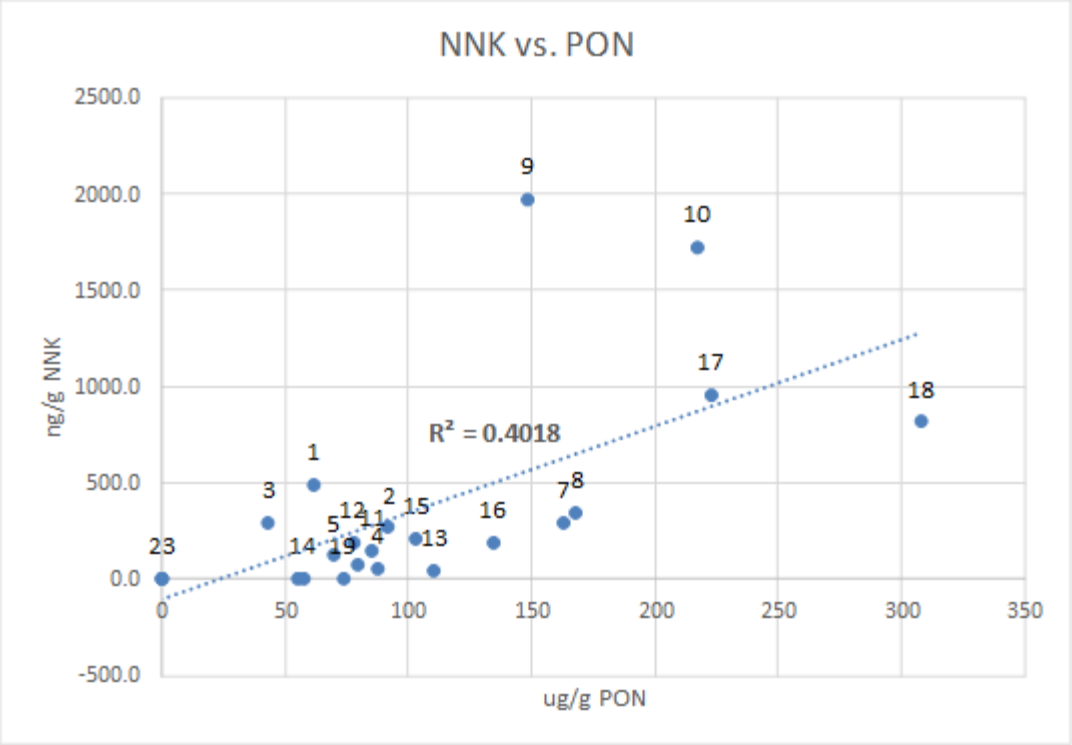
No.	Sample	PON mg/g	RSD%	Nic-Ox mg/g	RSD%	NNK ng/g	RSD%
16	FC U-Prod	134.3	0.4	215.6	0.6	188.6	0.28
17	Bu L-Prod	223.0	0.7	239.6	0.4	958.8	4.45
18	Bu U-Prod	308.4	0.6	252.0	0.8	819.5	2.91
19	O1-Prod	73.5	0.2	163.7	0.4	7.6	3.06
20	O2-Prod	55.4	0.5	148.1	0.7	1.3	5.22
21	FC1-LN	BDL*	-	BDL	-	0.2	7.25
22	FC2-LN	BDL	-	BDL	-	0.0	-
23	Bu-LN	BDL	-	BDL	-	0.2	13.00
24	Moist 1	10.5	1.4	36.5	1.6	431.8	3.19
25	Moist 2	6.6	1.8	48.8	0.9	365.7	1.10
26	Moist 3	11.5	1.6	153.8	0.2	523.4	3.23
27	Moist 4	21.2	0.7	176.9	1.1	1126.2	6.10
28	Moist 5	11.6	1.2	185.5	0.5	662.5	3.04
29	Moist 6	10.9	0.0	271.1	1.0	806.1	2.13
30	Moist 7	16.6	1.1	58.6	1.6	659.1	1.10
31	Moist 8	6.9	0.4	61.0	2.8	456.5	2.56

* BDL = below detection limit

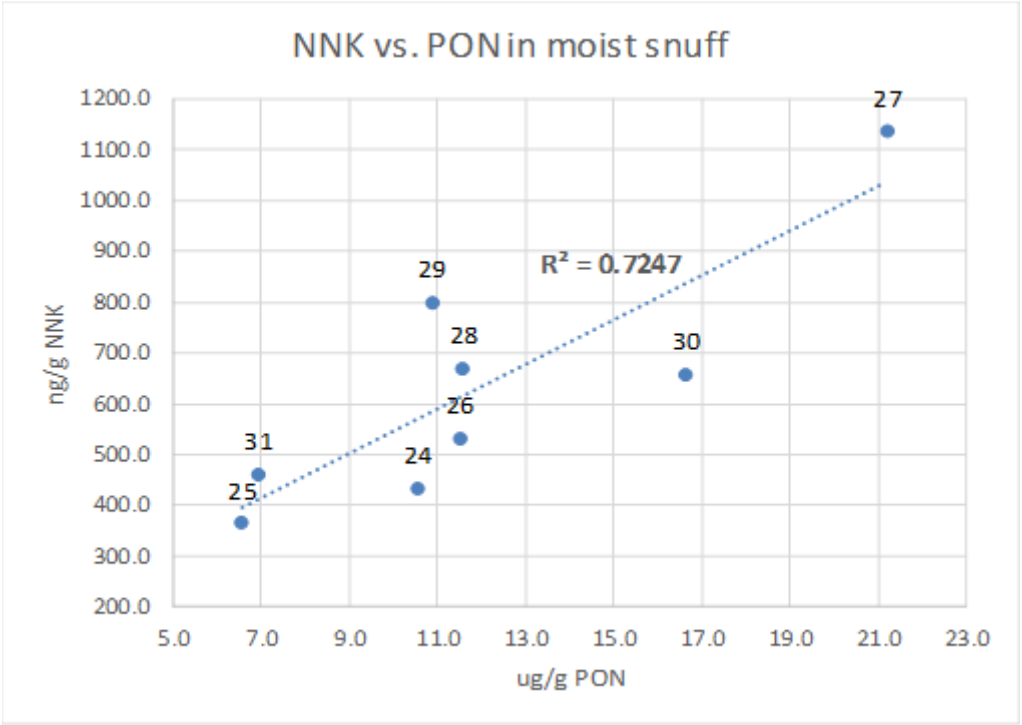
Correlation between NNK levels and PON levels in all samples



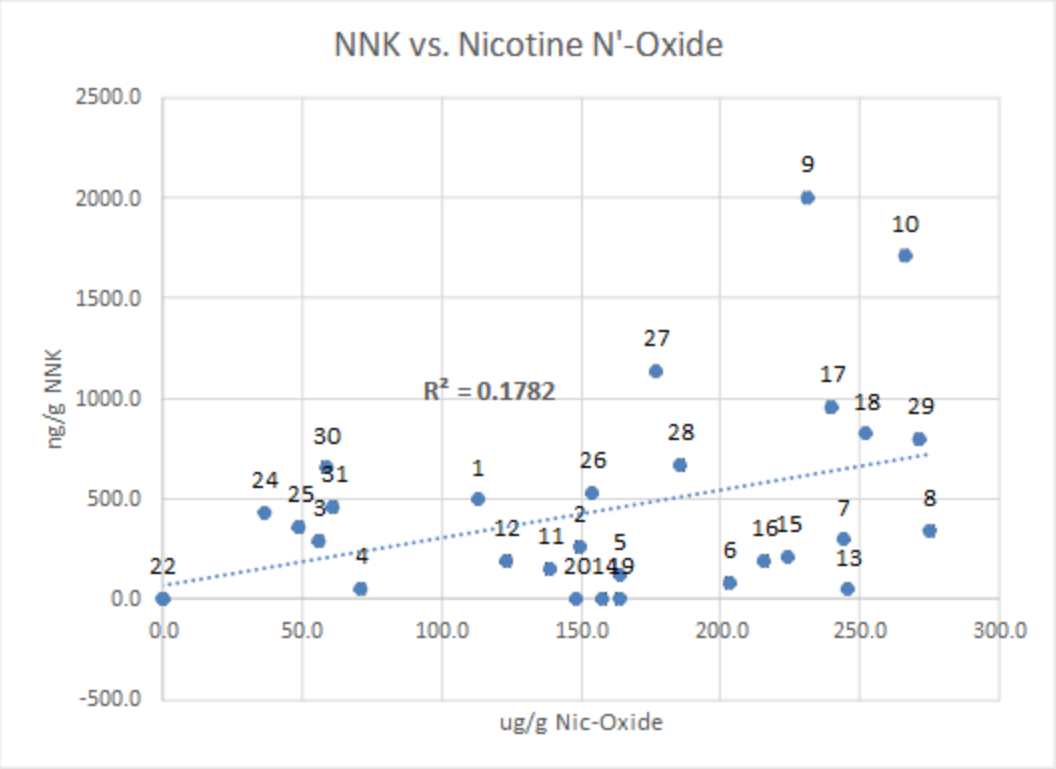
Correlation between NNK levels and PON levels only in tobacco samples



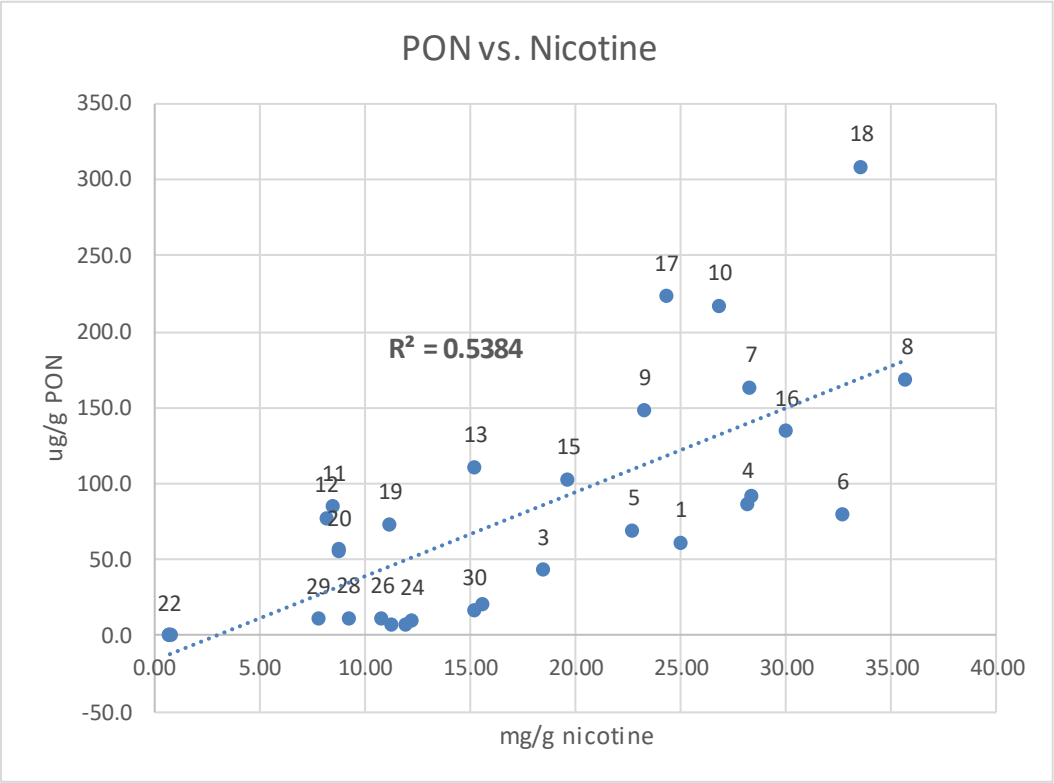
Correlation between NNK levels and PON levels only in moist snuff samples



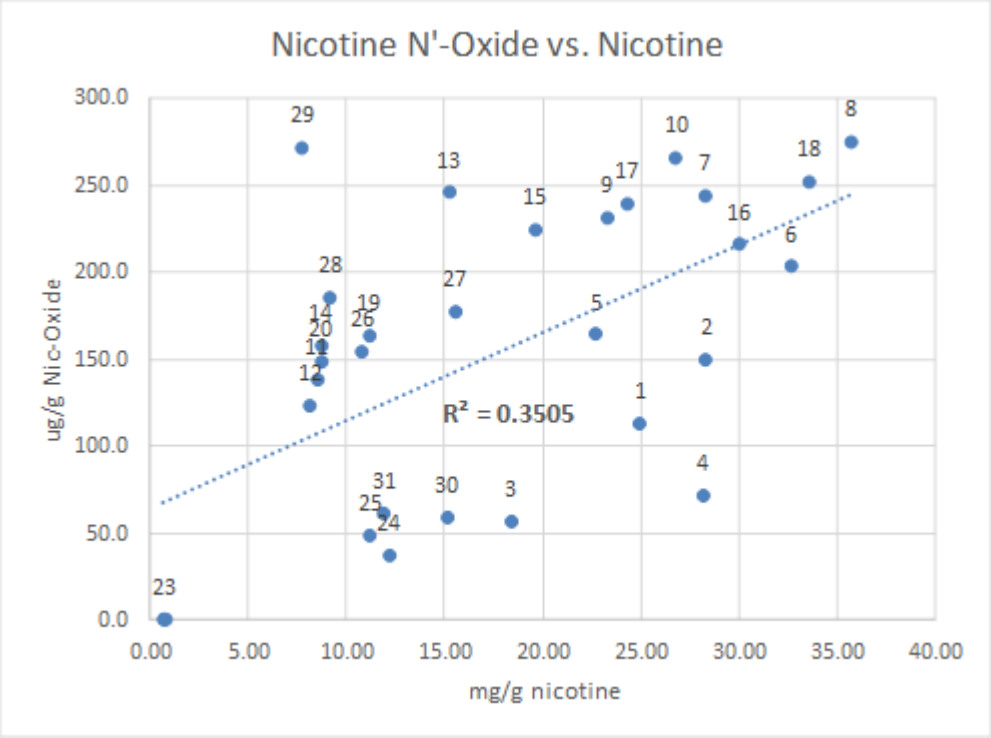
Correlation between NNK levels and Nic-Ox levels



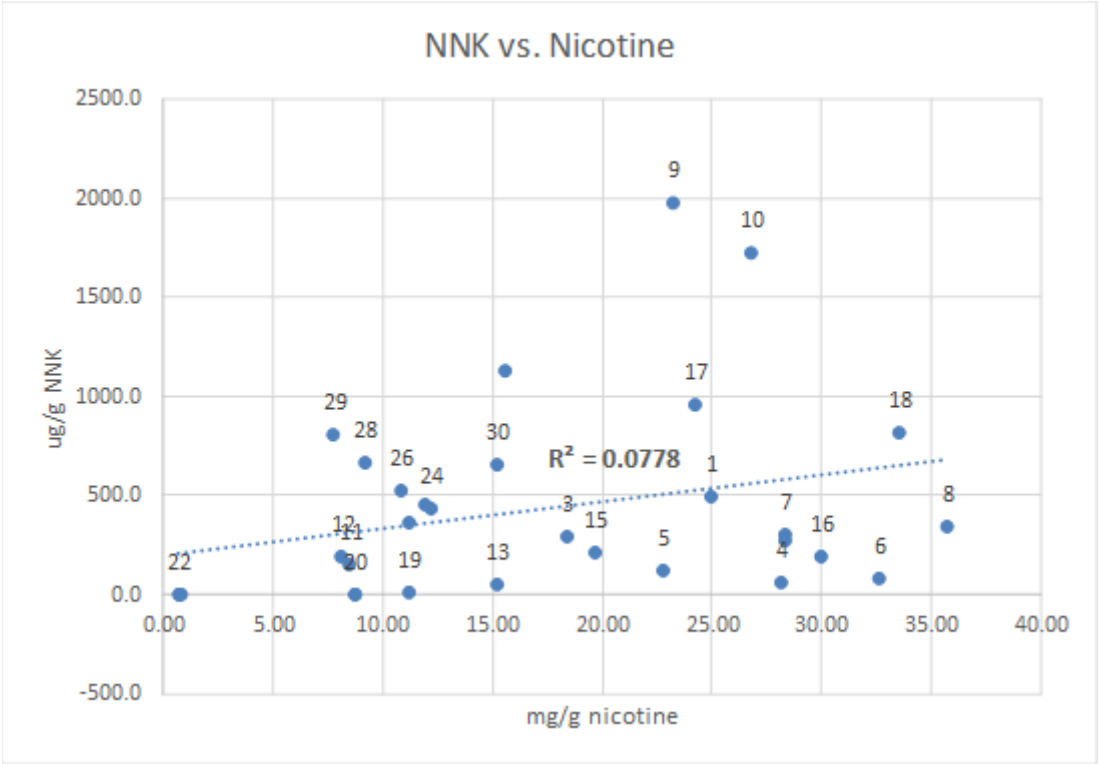
Correlation between PON and nicotine levels



Correlation between Nic-Ox and nicotine levels



Correlation between NNK and nicotine levels



Conclusions

- A new method for the analysis of PON and Nic-Ox has been developed and was used for the analysis of these compounds in several tobaccos and moist snuff samples.
- A previously developed method for the analysis of TSNAs was used for the analysis of NNK in the same samples.
- Nicotine was also measured in these samples using a LC-UV procedure.
- The hypothesis that the levels of NNK in tobacco depends on the PON levels has been tested attempting correlations between PON, Nic-Ox, nicotine, and NNK.
- All the attempted correlations indicated very low R^2 correlation coefficients.
- The study did not prove that NNK in tobacco or moist snuff is not generated via PON, but it demonstrated that the limiting factor (rate limiting step) in the formation of NNK in tobacco and moist snuff is not the level of PON or that of Nic-Ox.